FINAL

BIOAVAILABILITY OF LEAD IN TEST MATERIALS FROM THE VB-I70 SITE - DENVER, COLORADO

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EXECUTIVE SUMMARY

A study using young swine as test animals was performed to measure the gastrointestinal absorption of lead in two soils from the Vasquez Boulevard/I-70 site (VB-I70) located in Denver, Colorado. Young swine were selected for use in the study primarily because the gastrointestinal physiology and overall size of young swine are similar to that of young children, who are the population of prime concern for exposure to soil lead.

The test materials were prepared by combining soil samples collected from residential properties within the study area. The soil samples were selected to represent both an Eastern and Western area. The lead concentration in these samples was 723 parts per million (ppm) for test material #1 (Eastern Sample) and 987 ppm for test material #2 (Western Sample). Groups of 5 swine were given average oral doses of 103.7, 311.2 or 691.6 mg/kg-d of test material #1 or 76.0, 228.0, or 506.6 mg/kg-d of test material #2 for 15 days. This corresponded to target average doses of 75, 225, or 500 ug/kg/day of lead. Other groups of animals were given a standard lead reference material (lead acetate) orally at doses of 0, 25, 75, or 225 ug Pb/kg-day. The amount of lead absorbed by each animal was evaluated by measuring the amount of lead in the blood (measured on days 0, 1, 2, 3, 5, 7, 9, 12, and 15), and the amount of lead in liver, kidney and bone (measured on day 15 at study termination). The amount of lead present in blood or tissues of animals exposed to test soils was compared to that for animals exposed to lead acetate, and the results were expressed as relative bioavailability (RBA). For example, a relative bioavailability of 50% means that 50% of the lead in soil was absorbed equally as well as lead from lead acetate, and 50% behaved as if it were not available for absorption. Thus, if lead acetate were 40% absorbed, the test material would be 20% absorbed.

The RBA results for the two samples from the VB-I70 site are summarized below:

Measurement Endpoint	Test Material #1 Eastern Sample	Test Material #2 Western Sample
Blood Lead Area Under Curve	87%	85%
Liver Lead	98%	70%
Kidney Lead	97%	78%
Bone Lead	69%	56%

Because the estimates of RBA based on blood, liver, kidney, and bone do not agree in all cases, judgment must be used in interpreting the data. In general, EPA recommends greatest emphasis be placed on the RBA estimates derived from the blood lead data. This is because blood lead data are more robust and less susceptible to random errors than the tissue lead data, so there is greater confidence in RBA estimates based on blood lead. In addition, absorption into the central compartment is an early indicator of lead exposure, is the most relevant index

of central nervous system exposure, and is the standard measurement endpoint in investigations of this sort. However, data from the tissue endpoints (liver, kidney, bone) also provide valuable information. EPA considers the plausible range to extend from the RBA based on blood AUC to the mean of the other three tissues (liver, kidney, bone). The preferred range is the interval from the RBA based on blood to the mean of the blood RBA and the tissue mean RBA. Our suggested point estimate is the mid-point of the preferred range. These values are presented below:

Relative Bioavailability of Lead	Test Material #1 Eastern Sample	Test Material #2 Western Sample
Plausible Range	87-88%	68-85%
Preferred Range	87-88%	76-85%
Suggested Point Estimate	87%	81%

These RBA estimates may be used to help assess lead risk at this site by refining the estimate of absolute bioavailability (ABA) of lead in soil, as follows:

$$ABA_{soil} = ABA_{soluble} * RBA_{soil}$$

Available data indicate that fully soluble forms of lead are about 50% absorbed by a child. Thus, the estimated absolute bioavailability of lead in the site samples is as follows:

Absolute Bioavailability of Lead	Test Material #1 Eastern Sample	Test Material #2 Western Sample
Plausible Range	43-44%	34-42%
Preferred Range	43-44%	38-42%
Suggested Point Estimate	43%	40%

These absolute bioavailability estimates are appropriate for site-specific use in EPA's IEUBK model, although it is clear that there is both natural variability and uncertainty associated with these estimates. This variability and uncertainty arises from several sources, including: 1) the inherent variability in the responses of different individual animals to lead exposure, 2) uncertainty in the relative accuracy and applicability of the different measurement endpoints, 3) the extrapolation of measured RBA values in swine to young children, and 4) the potential effect of food in the stomach on lead absorption. Thus, the values reported above are judged to be reasonable estimates of typical lead absorption by children at this site, but should be interpreted with the understanding that the values are not certain.

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1.0 INTRODUCTION

Absolute and Relative Bioavailability

Bioavailability is a concept that relates to the absorption of chemicals and how absorption depends upon the physical-chemical properties of the chemical and its medium (e.g., dust, soil, rock, food, water, etc.) and the physiology of the exposed receptor. Bioavailability is normally described as the fraction (or percentage) of a chemical which enters into the blood following an exposure of some specified amount, duration and route (usually oral). In some cases, bioavailability may be measured using chemical levels in peripheral tissues such as liver, kidney, and bone, rather than blood. The fraction or percentage absorbed may be expressed either in absolute terms (absolute bioavailability, ABA) or in relative terms (relative bioavailability, RBA). **Absolute bioavailability** is measured by comparing the amount of chemical entering the blood (or other tissue) following oral exposure to test material with the amount entering the blood (or other tissue) following intravenous exposure to an equal amount of some dissolved form of the chemical. Similarly, **relative bioavailability** is measured by comparing oral absorption of test material to oral absorption of some fully soluble form of the chemical (e.g., either the chemical dissolved in water, or a solid form that is expected to fully dissolve in the stomach). For example, if 100 ug of dissolved lead were administered in drinking water and a total of 50 ug entered the blood, the ABA would be 0.50 (50%). Likewise, if 100 ug of lead in soil were administered and 30 ug entered the blood, the ABA for soil would be 0.30 (30%). If the lead dissolved in water were used as the reference substance for describing the relative amount of lead absorbed from soil, the RBA would be 0.30/0.50 = 0.60 (60%). These values (50% absolute bioavailability of dissolved lead and 30% absolute absorption of lead in soil) are the values currently employed as defaults in EPA's IEUBK model.

It is important to recognize that simple solubility of a test material in water or some other fluid (e.g., a weak acid intended to mimic the gastric contents of a child) may not be a reliable estimator of bioavailability due to the non-equilibrium nature of the dissolution and transport processes that occur in the gastrointestinal tract (Mushak 1991). For example, fluid volume and pH are likely to be changing as a function of time, and transport of lead across the gut will prevent an approach to equilibrium concentrations, especially for poorly soluble lead compounds. However, information on the solubility of lead in different materials is useful in interpreting the importance of solubility as a determinant of bioavailability. To avoid confusion, the term "bioaccessability" is used to refer to the relative amount of lead that dissolves under a specified set of test conditions.

For additional discussion about the concept and application of bioavailability see Goodman et al. (1990), Klaassen et al. (1996), and/or Gibaldi and Perrier (1982).

Using Bioavailability Data to Improve Exposure Calculations for Lead

When data are available on the bioavailability of lead in soil, dust, or other soil-like waste material at a site, this information can often be used to improve the accuracy of exposure and risk calculations at that site. The basic equation for estimating the site-specific ABA of a test soil is as follows:

$$ABA_{soil} = ABA_{soluble} \cdot RBA_{soil}$$

where:

ABA_{soil}	=	Absolute bioavailability of lead in soil ingested by a child
$ABA_{soluble}$	=	Absolute bioavailability in children of some dissolved or fully soluble form of lead
RBA_{soil}	=	RBA for soil measured in swine

Based on available information on lead absorption in humans and animals, the EPA estimates that the absolute bioavailability of lead from water and other fully soluble forms of lead is usually about 50% in children. Thus, when a reliable site-specific RBA value for soil is available, it may be used to estimate a site-specific absolute bioavailability as follows:

$$ABA_{soil} = 50\% \cdot RBA_{soil}$$

In the absence of site-specific data, the absolute absorption of lead from soil, dust and other similar media is estimated by EPA to be about 30%. Thus, the default RBA used by EPA for lead in soil and dust compared to lead in water is 30%/50% = 60%. When the measured RBA in soil or dust at a site is found to be less than 60% compared to some fully soluble form of lead, it may be concluded that exposures to and risks from lead in these media at that site are probably lower than typical default assumptions. If the measured RBA is higher than 60%, absorption of and risk from lead in these media may be higher than usually assumed.

2.0 STUDY DESIGN

A standardized study protocol for measuring absolute and relative bioavailability of lead was developed based upon previous study designs and investigations that characterized the young pig model (Weis et al. 1995). The study was performed as nearly as possible within the spirit and guidelines of Good Laboratory Practices (GLP: 40 CFR 792). Standard Operating Procedures (SOPs) that included detailed methods for all aspects of the study were prepared, approved, and distributed to all study members prior to the study (USEPA 2000).

2.1 Test Materials

Soil samples were collected from various residential properties within the VBI70 site which were selected for specific concentrations of lead and arsenic (Washington Group, 2000; attached as appendix B). The two soils were prepared to represent both Eastern (Test Material #1) and Western (Test Material #2) neighborhoods within this site. Six individual soil samples were combined to make the Eastern sample, and five individual soil samples were combined to make the Western sample. Each sample was dried in a laboratory over at 105 C, bulk sieved with a 2-mm screen and fine sieved with a 250-mm screen. Further details regarding the selection and preparation of test materials can be found in a separate technical memorandum (Washington Group, 2000).

Table 2-1 summarizes the lead and arsenic content of the test soils measured using ICP method SW6010. As seen, average lead concentrations in the Eastern and Western Test Materials are 723 and 987 mg/kg, respectively.

TABLE 2-1 LEAD AND ARSENIC ANALYSIS OF TEST MATERIALS

Sample	Replicate	Arsenic (mg/kg)	Lead (mg/kg)
Eastern Sample (TM#1)	#1	19	700
	#2	19	710
	#3	20	760
	Average	**19**	**723**
Western Sample (TM#2)	#1	26	970
	#2	25	1000
	#3	24	990
	Average	**25**	**987**

Each sample of test material was well mixed and analyzed by electron microprobe in order to identify a) how frequently particles of various lead minerals were observed, b) how frequently different types of mineral particles occur entirely inside particles of rock or slag ("included") and how often they occur partially or entirely outside rock or slag particles ("liberated"), c) the size distribution of particles of each mineral class, and d) approximately how much of the total amount of lead in the sample occurs in each mineral type. This is referred to as "relative lead mass". The results are summarized in Figures 2-1 to 2-3.

As seen in Figure 2-1 (top panel), the most common lead-bearing particle type (i.e., those which are observed most often) for the Eastern Sample (Test Material #1) was Iron Oxide, accounting for about 34% of all lead-bearing particles. However, as shown in Figure 2-2 (upper panel)), because the concentration of lead in iron oxide is relatively low, this phase accounted for only about 7.3% of the lead mass in this sample. The remainder of the lead in the eastern sample occurred mainly in particles of phosphate (41.4%), anglesite (15.6%) and paint (12%). Also shown in Figure 2-1 (bottom panel) are the results for the Western Sample (Test Material #2). As seen, the most common lead-bearing particle types were slag, phosphate, organics and iron oxide, accounting for about 18.3%, 17.5%, 18.8% and 16.7% of all lead-bearing particles, respectively. The majority of lead mass (Figure 2-2, bottom panel) in this sample was found in the phosphate (52.7%) and cerussite (18.3%) phases.

Figure 2-3 shows the distribution of the size of lead-bearing particles in the sample. As seen, there was a fairly broad distribution of lead-bearing particle sizes in both test materials, mainly ranging from 50-200 um. As noted above, small particles are often assumed to be more likely to adhere to the hand and be ingested and/or transported into the house. Further, small particles have larger surface area-to-volume ratios than larger particles, and so may tend to dissolve more rapidly in the acidic contents of the stomach than larger particles. Thus, small particle (e.g., less than 50-100 um) are thought to be of greater potential concern to humans than larger particles (e.g., 100 -250 um or larger).

Another property of lead particles that may be important in determining bioaccessability and/or bioavailability is the degree to which they are partially or entirely free from surrounding matrix ("liberated"). Based on the measured frequency of each type of particle existing in a liberated state, it can be calculated that of the total relative lead present in the samples, about 97.2% exists in liberated particles in the Eastern Sample (TM1) and 95.5% exists in liberated particles in the Western Sample (TM2). These high percentages of partially or entirel liberated grains may tend to increase the bioavailability of lead in the samples.

FIGURE 2-1 - FREQUENCY OF LEAD PARTICLES

FIGURE 2-2 LEAD MASS

FIGURE 2-3 PARTICLE SIZE DISTRIBUTION

2.2 Experimental Animals

Young swine were selected for use in these studies because they are considered to be a good physiological model for gastrointestinal absorption in children (Weis and LaVelle 1991). The animals were intact males of the Pig Improvement Corporation (PIC) genetically defined Line 26, and were purchased from Chinn Farms, Clarence, MO. The animals were held under quarantine to observe their health for one week before beginning exposure to test materials. To minimize weight variations between animals and groups, the number of animals purchased from the supplier was six more than needed for the study, and the six animals most different in body weight on day -4 (either heavier or lighter) were excluded from further study. The remaining animals were assigned to dose groups at random. When exposure began (day zero), the animals were about 5-6 weeks old (juveniles, weaned at 3 weeks) and weighed an average of about 9.7 kg. Animals were weighed every three days during the course of the study. The group mean body weights over the course of the study are shown in Figure 2-4. On average, animals gained about 0.5 kg/day, and the rate of weight gain was comparable in all groups.

All animals were housed in individual lead-free stainless steel cages. Each animal was examined by a certified veterinary clinician (swine specialist) prior to being placed on study, and all animals were examined daily by an attending veterinarian while on study. Blood samples were collected for hematological analysis on days -4, 7, and 15 to assist in clinical health assessments. In this study, there was one animal that was removed from the study due to concerns over poor health.

2.3 Diet

Animals provided by the supplier were weaned onto standard pig chow purchased from MFA Inc., Columbia, MO. In order to minimize lead exposure from the diet, the animals were gradually transitioned from the MFA feed to a special low-lead feed (guaranteed less than 0.2 ppm lead, purchased from Zeigler Brothers, Inc., Gardners, PA) over the time interval from day -7 to day -3, and this feed was then maintained for the duration of the study. The feed was nutritionally complete and met all requirements of the National Institutes of Health-National Research Council. The typical nutritional components and chemical analysis of the feed is presented in Table 2-2. Typically, the feed contained approximately 5.7% moisture, 1.7% fiber, and provided about 3.4 kcal of metabolizable energy per gram. Analysis of two feed samples during this experiment indicated the mean lead level was 0.15 ppm.

Each day every animal was given an amount of feed equal to 5% of the mean body weight of all animals on study. Feed was administered in two equal portions of 2.5% of the mean body weight at each feeding. Feed was provided at 11:00 AM and 5:00 PM daily. Drinking water was provided ad libitum via self-activated watering nozzles within each cage. Analysis of samples from randomly selected drinking water nozzles indicated the mean lead concentration (treating non-detects at one-half the quantitation limit) was less than 1 ug/L.

FIGURE 2-4 BODY WEIGHTS

TABLE 2-2 TYPICAL FEED COMPOSITION[a]

Nutrient Name	Amount	Nutrient Name	Amount
Protein	20.1021%	Chlorine	0.1911%
Arginine	1.2070%	Magnesium	0.0533%
Lysine	1.4690%	Sulfur	0.0339%
Methionine	0.8370%	Manganese	20.4719 ppm
Met+Cys	0.5876%	Zinc	118.0608 ppm
Tryptophan	0.2770%	Iron	135.3710 ppm
Histidine	0.5580%	Copper	8.1062 ppm
Leucine	1.8160%	Cobalt	0.0110 ppm
Isoleucine	1.1310%	Iodine	0.2075 ppm
Phenylalanine	1.1050%	Selenium	0.3196 ppm
Phe+Tyr	2.0500%	Nitrogen Free Extract	60.2340%
Threonine	0.8200%	Vitamin A	5.1892 kIU/kg
Valine	1.1910%	Vitamin D3	0.6486 kIU/kg
Fat	4.4440%	Vitamin E	87.2080 IU/kg
Saturated Fat	0.5590%	Vitamin K	0.9089 ppm
Unsaturated Fat	3.7410%	Thiamine	9.1681 ppm
Linoleic 18:2:6	1.9350%	Riboflavin	10.2290 ppm
Linoleic 18:3:3	0.0430%	Niacin	30.1147 ppm
Crude Fiber	3.8035%	Pantothenic Acid	19.1250 ppm
Ash	4.3347%	Choline	1019.8600 ppm
Calcium	0.8675%	Pyridoxine	8.2302 ppm
Phos Total	0.7736%	Folacin	2.0476 ppm
Available Phosphorous	0.7005%	Biotin	0.2038 ppm
Sodium	0.2448%	Vitamin B12	23.4416 ppm
Potassium	0.3733%		

[a] Nutritional values provided by Zeigler Bros., Inc.

2.4 Dosing

The protocol for exposing animals to lead is shown in Table 2-3. The dose levels for lead acetate were based on experience from previous swine investigations that showed that doses of 25-225 ug Pb/kg/day gave clear and measurable increases in lead levels in all endpoints measured (blood, liver, kidney, bone). The doses of test materials were set at the same level as lead acetate, with one higher dose (500 ug Pb/kg-day) included in case the test materials were found to yield very low responses.

Animals were exposed to lead acetate or test material for 15 days, with the dose for each day being administered in two equal portions given at 9:00 AM and 3:00 PM (two hours before feeding). Doses were based on measured group mean body weights, and were adjusted every three days to account for animal growth. For animals exposed by the oral route, dose material was placed in the center of a small portion (about 5 grams) of moistened feed, and this was administered to the animals by hand. In this study, all doses were consumed by the animals without delay or spillage. However, on day 3, one animal in Group 8 was inadvertently given a dose for Group 9 in addition to it's own dose during the morning dosing. This dosing discrepancy was accounted for in further calculations.

Actual mean doses, calculated from the administered doses and the measured body weights, are also shown in Table 2-3.

2.5 Collection of Biological Samples

Samples of blood were collected from each animal on the first day of exposure (day 0), and on days 1, 2, 3, 5, 7, 9, 12, and 15 following the start of exposure. All blood samples were collected by vena-puncture of the anterior vena cava, and samples were immediately placed in purple-top Vacutainer® tubes containing EDTA as anticoagulant. Although EDTA is a chelator of metals, its presence in the sampling tubes will not impact the analytical results for lead. This is because the nitric acid digest used in the analysis destroys the organic constituents in the blood, thereby freeing all lead for analysis. Blood samples were collected each sampling day beginning at 8:00 AM, approximately one hour before the first of the two daily exposures to lead on the sampling day and 17 hours after the last lead exposure the previous day. This blood collection time was selected because the rate of change in blood lead resulting from the preceding exposures is expected to be relatively small after this interval (LaVelle et al. 1991, Weis et al. 1993), so the exact timing of sample collection relative to last dosing is not likely to be critical.

Following collection of the final blood sample at 8:00 AM on day 15, all animals were humanely euthanized and samples of liver, kidney and bone (the right femur) were removed and stored in lead-free plastic bags for lead analysis. Samples of all biological samples collected were archived in order to allow for reanalysis and verification of lead levels, if needed. All animals were also subjected to detailed examination at necropsy by a certified veterinary pathologist in order to assess overall animal health.

TABLE 2-3 DOSING PROTOCOL

Group	Number of Animals	Dose Material Administered	Exposure Route	Lead Dose (ug Pb/kg-d)	
				Target	Actual[a]
1	5	Lead Acetate	Oral	25	26.0
2	5	Lead Acetate	Oral	75	78.0
3	5	Lead Acetate	Oral	225	233.1
4	5	Eastern Sample (TM#1)	Oral	75	77.2
5	5	Eastern Sample (TM#1)	Oral	225	232.0
6	5	Eastern Sample (TM#1)	Oral	500	513.1
7	5	Western Sample (TM#2)	Oral	75	77.1
8	5	Western Sample (TM#2)	Oral	225	236.3
9	5	Western Sample (TM#2)	Oral	500	511.5
10	3[b]	Control	Oral	0	0

Doses were administered in two equal portions given at 9:00 AM and 3:00 PM each day. Doses were based on the mean weight of the animals in each group, and were adjusted every three days to account for weight gain.

[a] Calculated as the administered daily dose divided by the measured or extrapolated daily body weight, averaged over days 0-14 for each animal and each group.

[b] Three control animals were used in this study due to contraints on facility size. Based on previous investigations, this approach resulted in reliable results.

2.6 Preparation of Biological Samples for Analysis

Blood

One mL of whole blood was removed from the purple-top Vacutainer and added to 9.0 mL of "matrix modifier", a solution recommended by the Centers for Disease Control and Prevention (CDCP) for analysis of blood samples for lead. The composition of matrix modifier is 0.2% (v/v) ultrapure nitric acid, 0.5% (v/v) Triton X-100, and 0.2% (w/v) dibasic ammonium phosphate in deionized and ultrafiltered water. Samples of the matrix modifier were routinely analyzed for lead to ensure the absence of lead contamination.

Liver and Kidney

One gram of soft tissue (liver or kidney) was placed in a lead-free screw-cap teflon container with 2 mL of concentrated (70%) nitric acid and heated in an oven to 90°C overnight. After cooling, the digestate was transferred to a clean lead-free 10 mL volumetric flask and diluted to volume with deionized and ultrafiltered water.

Bone

The right femur of each animal was removed and defleshed, and dried at 100°C overnight. The dried bones were then placed in a muffle furnace and dry-ashed at 450°C for 48 hours. Following dry ashing, the bone was ground to a fine powder using a lead-free mortar and pestle, and 200 mg was removed and dissolved in 10.0 mL of 1:1 (v:v) concentrated nitric acid/water. After the powdered bone was dissolved and mixed, 1.0 mL of the acid solution was removed and diluted to 10.0 mL by addition of 0.1% (w/v) lanthanum oxide (La_2O_3) in deionized and ultrafiltered water.

2.7 Lead Analysis

Samples of biological tissue (blood, liver, kidney, bone) and other materials (food, water, reagents and solutions, etc.) were arranged in a random sequence and provided to the analytical laboratory in a blind fashion (identified to the laboratory only by a chain of custody tag number). Each sample was analyzed for lead using a Perkin Elmer Model 5100 graphite furnace atomic absorption spectrophotometer. Internal quality assurance samples were run every tenth sample, and the instrument was recalibrated every 15th sample. A blank, duplicate and spiked sample were run every 20th sample.

All results from the analytical laboratory were reported in units of ug Pb/L of prepared sample. The quantitation limit was defined as three-times the standard deviation of a set of seven replicates of a low-lead sample (typically about 2-5 ug/L). The standard deviation was usually about 0.3 ug/L, so the quantitation limit was usually about 0.9-1.0 ug/L (ppb). For prepared blood samples (diluted 1/10), this corresponds to a quantitation limit of 10 ug/L (1 ug/dL). For soft tissues (liver and kidney, diluted 1/10), this corresponds to a quantitation limit of 10 ug/kg (ppb) wet weight, and for bone (final dilution = 1/500) the corresponding quantitation limit is 0.5 ug/g (ppm) ashed weight.

3.0 DATA ANALYSIS

3.1 Overview

Studies on the absorption of lead are often complicated because some biological responses to lead exposure may be non-linear functions of dose (i.e., tending to flatten out or plateau as dose increases). The cause of this non-linearity is uncertain but might be due either to non-linear **absorption kinetics** and/or to non-linear **biological response** per unit dose absorbed. When the dose-response curve for either the reference material (lead acetate) and/or the test material is non-linear, RBA is equal to the ratio of doses that produce equal responses (not the ratio of responses at equal doses). This is based on the simple but biologically plausible assumption that equal absorbed doses yield equal biological responses. Applying this assumption leads to the following general methods for calculating RBA from a set of non-linear experimental data:

1. Plot the biological responses of individual animals exposed to a series of oral doses of soluble lead (e.g., lead acetate). Fit an equation which gives a smooth line through the observed data points.

2. Plot the biological responses of individual animals exposed to a series of doses of test material. Fit an equation which gives a smooth line through the observed data.

3. Using the best fit equations for reference material and test material, calculate RBA as the ratios of doses of test material and reference material which yield equal biological responses. Depending on the relative shape of the best-fit lines through the lead acetate and test material dose response curves, RBA may either be constant (dose-independent) or variable (dose-dependent).

The principal advantage of this approach is that it is not necessary to understand the basis for a non-linear dose response curve (non-linear absorption and/or non-linear biological response) in order to derive valid RBA estimates. Also, it is important to realize that this method is very general, as it will yield correct results even if one or both of the dose-response curves are linear. In the case where both curves are linear, RBA is dose-independent and is simply equal to the ratio of the slopes of the best-fit linear equations.

3.2 Fitting the Curves

There are a number of different mathematical equations which can yield reasonable fits with the dose-response data sets obtained in this study. Conceptually, any equation which gives a smooth fit would be acceptable, since the main purpose is to allow for interpolation of responses between test doses. In selecting which equations to employ, the following principles were applied: 1) mathematically simple equations were preferred over mathematically complex equations, 2) the shape of the curves had to be smooth and biologically realistic, without inflection points, maxima or minima, and 3) the general form of the equations had to be able

to fit data not only from this one study, but from all the studies that are part of this project. After testing a wide variety of different equations, it was found that all data sets could be well fitted using one of the following three forms:

Linear (LIN):	Response = a + b·Dose
Exponential (EXP):	Response = a + c·(1-exp(-d·Dose))
Combination (LIN+EXP):	Response = a + b·Dose + c·(1-exp(-d·Dose))

Although underlying mechanism was not considered in selecting these equations, the linear equation allows fitting data that do not show evidence of saturation in either uptake or response, while the exponential and mixed equations allow evaluation of data that appear to reflect some degree of saturation in uptake and/or response.

Each dose-response data set was fit to each of the equations above. If one equation yielded a fit that was clearly superior (as judged by the value of the adjusted correlation coefficient R^2) to the others, that equation was selected. If two or more models fit the data approximately equally well, then the simplest model (that with the fewest parameters) was selected. In the process of finding the best-fits of these equations to the data, the values of the parameters (a, b, c, and d) were subjected to some constraints, and some data points (those that were outside the 95% prediction limits of the fit) were excluded. These constraints and outlier exclusion steps are detailed in Appendix A (Section 3). In general, most blood lead AUC dose-response curves were best fit by the exponential equation, and most dose-response curves for liver, kidney and bone were best fit by linear equations. In evaluating spleen results, it was determined that data were best fit by the exponential equation.

3.3 Responses Below Quantitation Limit

In some cases, most or all of the responses in a group of animals were below the quantitation limit for the endpoint being measured. For example, this was normally the case for blood lead values in unexposed animals (both on day -4 and day 0), and in control animals. In these cases, samples were assigned a response equal to one-half the quantitation limit.

3.4 Quality Assurance

A number of steps were taken throughout this study and the other studies in this project to ensure the quality of the results. These steps are summarized below.

Duplicates

A randomly selected set of about 5% of all samples generated during the study were submitted to the laboratory in a blind fashion for duplicate analysis. The raw data are presented in

Appendix A, and Figure 3-1 plots the results for blood (Panel A, upper) and for bone, liver, and kidney (Panel B, lower).

FIGURE 3-1 QA DUPS

As seen, there was good intra-laboratory reproducibility between duplicate samples for both blood and tissues, with linear regression lines having a slope near 1.0, an intercept near zero, and an R^2 value near 1.0. One blood sample (not represented in the graph) was determined to be an outlier (original value 6.5; duplicate value 28.7).

Standards

The Centers for Disease Control and Prevention (CDCP) provides a variety of blood lead "check samples" for use in quality assurance programs for blood lead studies. Each time a group of blood samples was prepared and sent to the laboratory for analysis, several CDCP check samples of different concentrations were included in random order and in a blind fashion.

The results for the samples submitted during this study are presented in Appendix A, and the values are plotted in Figure 3-2. For the "low" standard (nominal = 1.7 ug/dL), the average measured value was 2.0 ug/dL. For the "medium" and "high" standards, the means of the measured values were 3.7 ug/dL (nominal = 4.8 ug/dL) and 13.6 ug/dL (nominal = 14.9 ug/dL).

Data Audits and Spreadsheet Validation

All analytical data generated by EPA's analytical laboratory were validated prior to being released in the form of a database file. These electronic data files were "decoded" (linking the sample tag to the correct animal and day) using Microsoft's database system ACCESS®. To ensure that no errors occurred in this process, original electronic files were printed out and compared to printouts of the tag assignments and the decoded data.

All spreadsheets used to manipulate the data and to perform calculations (see Appendix A) were validated by hand-checking random cells for accuracy.

FIGURE 3-2 CHECK STANDARDS

4.0 RESULTS

The following sections provide results based on the group means for each dose group investigated in this study. Appendix A provides detailed data for each individual animal. Results from this study will be compared and contrasted with the results from other studies in a subsequent report.

4.1 Blood Lead vs Time

Figure 4-1 shows the group mean blood lead values as a function of time during the study. As seen, blood lead values began at or below quantitation limits (about 1 ug/dL) in all groups, and remained at or below quantitation limits in control animals (Group 10). In animals given repeated oral doses of lead acetate (Groups 1-3), Eastern soil (Groups 4-6), or Western soil (Groups 7-9), blood levels began to rise within 1-2 days, and tended to plateau by the end of the study (day 15).

4.2 Dose-Response Patterns

Blood Lead

The measurement endpoint used to quantify the blood lead response was the area under the curve (AUC) for blood lead vs time (days 0-15). AUC was selected because it is the standard pharmacokinetic index of chemical uptake into the blood compartment, and is relatively insensitive to small variations in blood lead level by day. The AUC was calculated using the trapezoidal rule to estimate the AUC between each time point that a blood lead value was measured (days 0, 1, 2, 3, 5, 7, 9, 12, and 15), and summing the areas across all time intervals in the study. The detailed data and calculations are presented in Appendix A, and the results are shown graphically in Figure 4-2. Each data point reflects the group mean exposure and group mean response, with the variability in dose and response shown by standard error bars. The figure also shows the best-fit equation through each data set.

As seen, the dose response pattern is non-linear for both the soluble reference material (lead acetate, abbreviated "PbAc"), and for each of the two test soils. Dose response curves for both soils are similar to those seen for lead acetate.

Tissue Lead

The dose-response data for lead levels in bone, liver, and kidney (measured at sacrifice on day 15) are detailed in Appendix A, and are shown graphically in Figures 4-3 through 4-5, respectively. As seen, all of these dose response curves for tissues are fit by linear equations, both for lead acetate and each of the two test soils.

FIGURE 4-1 GROUP MEAN BLOOD VALUES

FIGURE 4-2 AUC

FIGURE 4-3 BONE

FIGURE 4-4 LIVER

FIGURE 4-5 KIDNEY

4.3 Calculated RBA Values

Relative bioavailability values were calculated for each test material for each measurement endpoint (blood AUC, bone, liver, kidney) using the method described in Section 3.0. The results are shown below:

Measurement Endpoint	Estimated RBA	
	Eastern Test Material #1	Western Test Material #2
Blood Lead AUC	0.87	0.85
Liver Lead	0.98	0.70
Kidney Lead	0.97	0.78
Bone Lead	0.69	0.56

Recommended RBA Values

For each test soil, the estimates of RBA based on blood, liver, kidney, and bone are generally similar, but do not agree exactly in all cases. In general, we recommend greatest emphasis be placed on the RBA estimates derived from the blood lead data. There are several reasons for this recommendation, including the following:

1) Blood lead calculations are based on multiple measurements over time, and so are statistically more robust than the single measurements available for tissue concentrations. Further, blood is a homogeneous medium, and is easier to sample than complex tissues such as liver, kidney and bone. Consequently, the AUC endpoint is less susceptible to random measurement errors, and RBA values calculated from AUC data are less uncertain.

2. Blood is the central compartment and one of the first compartments to be affected by absorbed lead. In contrast, uptake of lead into peripheral compartments (liver, kidney, bone) depend on transfer from blood to the tissue, and may be subject to a variety of toxicokinetic factors that could make bioavailability determinations more complicated.

3. The dose-response curve for blood lead is non-linear, similar to the non-linear dose-response curve observed in children (e.g., see Sherlock and Quinn 1986). Thus, the response of this endpoint is known to behave similarly in swine as in children, and it is not known if the same is true for the tissue endpoints.

4. Blood lead is the classical measurement endpoint for evaluating exposure and health effects in humans, and the health effects of lead are believed to be proportional to blood lead levels.

However, data from the tissue endpoints (liver, kidney, bone) also provide valuable information. We consider the plausible range to extend from the RBA based on blood AUC to the mean of the other three tissues (liver, kidney, bone). The preferred range is the interval from the RBA based on blood to the mean of the blood RBA and the tissue mean RBA. Our suggested point estimate is the mid-point of the preferred range. These values are presented below:

Relative Bioavailability of Lead	Test Material	
	Eastern Test Material #1	Western Test Material #2
Plausible Range	0.87-0.88	0.68-0.85
Preferred Range	0.87-0.88	0.76-0.85
Suggested Point Estimate	0.87	0.81

4.4 Estimated Absolute Bioavailability in Children

These RBA estimates may be used to help assess lead risk at this site by refining the estimate of absolute bioavailability (ABA) of lead in soil, as follows:

$$ABA_{soil} = ABA_{soluble} * RBA_{soil}$$

Available data indicate that fully soluble forms of lead are about 50% absorbed by a child (USEPA 1991, 1994). Thus, the estimated absolute bioavailability of lead in the site samples is calculated as follows:

$$ABA_{Site} = 50\% * RBA_{Site}$$

Based on the RBA values shown above, the estimated absolute bioavailability in children is as follows:

Absolute Bioavailability of Lead	Test Material	
	Eastern Test Material #1	Western Test Material #2
Plausible Range	0.43-0.44	0.34-0.42
Preferred Range	0.43-0.44	0.38-0.42
Suggested Point Estimate	0.44	0.40

4.5 Uncertainty

The bioavailability estimates above are subject to uncertainty that arises from several different sources. First, differences in physiological and pharmacokinetic parameters between

individual animals leads to variability in response, even when exposure is the same. Because of this inter-animal variability in the responses of different animals to lead exposure, there is mathematical uncertainty in the best fit dose-response curves for both lead acetate and test material. This in turn leads to uncertainty in the calculated values of RBA, because these are derived from the two best-fit equations. Second, there is uncertainty in how to weight the RBA values based on the different endpoints, and how to select a point estimate for RBA that is applicable to typical site-specific exposure levels. Third, there is uncertainty in the quantitative extrapolation of measured RBA values in swine to young children. Even though the immature swine is believed to be a useful and meaningful animal model for gastrointestinal absorption in children, it is possible that differences in stomach pH, stomach emptying time, and other physiological parameters may exist and that RBA values in swine may not be precisely equal to values in children. Finally, studies in humans reveal that lead absorption is not constant even within an individual, but varies as a function of many factors (mineral intake, health status, etc.). One factor that may be of special importance is time after the last meal, with the presence of food tending to reduce lead absorption. The values of RBA measured in this study are intended to estimate the maximum uptake that occurs when lead is ingested in the absence of food. Thus, these values may be somewhat conservative for chilcren who ingest lead along with food. The magnitude of this bias is not known, although preliminary studies in swine suggest the factor may be relatively minor.

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APPENDIX A

DETAILED DATA AND CALCULATIONS

APPENDIX A

DETAILED DATA SUMMARY

1.0 OVERVIEW

Performance of this study involved collection and reduction of a large number of data items. All of these data items and all of the data reduction steps are contained in a Microsoft Excel spreadsheet named "VB_LEAD.XLS". This file is intended to allow detailed review and evaluation of all aspects of this study.

The following sections of this Appendix present printouts of selected tables and graphs from the XLS file. These tables and graphs provide a more detailed documentation of the individual animal data and the data reduction steps performed in this study than was presented in the main text. Any additional details of interest to a reader can be found in the XLS spreadsheet.

2.0 RAW DATA AND DATA REDUCTION STEPS

2.1 Body Weights and Dose Calculations

Animals were weighed on day -1 (one day before exposure) and every three days thereafter during the course of the study. Doses of lead for the three days following each weighing were based on the group mean body weight, adjusted by addition of 1 kg to account for the expected weight gain over the interval. After completion of the experiment, body weights were estimated by interpolation for those days when measurements were not collected, and the actual administered doses (ug Pb/kg) were calculated for each day and then averaged across all days. If an animal missed a dose or was given an incorrect dose, the calculation of average dose corrected for these factors. These data and data reduction steps are shown in Tables A-1 and A-2. During this study, one animal in Group 8, was inadvertently administered a Group 9 doughball in addition to its assigned doughball during the morning dosing on Day 3. This misdosing was accounted for in the spreadsheets for this experiment.

2.2 Blood Lead vs Time

Blood lead values were measured in each animal on days 0, 1, 2, 3, 5, 7, 9, 12, and 15. The raw laboratory data (reported as ug/L of diluted blood) are shown in Table A-3. These data were adjusted as follows: a) non-detects were evaluated by assuming a value equal to one-half the quantitation limit, and b) the concentrations in diluted blood were converted to units of ug/dL in whole blood by dividing by a factor of 1 dL of blood per L of diluted sample. The results are shown in the right-hand column of Table A-3. Figures A-1 to A-3 plot the results for individual animals organized by group and by day. Figure A-4 plots the mean for each dosing group by day.

After adjustment as above, values that were more than a factor of 1.5 above or below the group mean for any given day were "flagged" by computer as potential outliers. These values are shown in Table A-4 by cells that are shaded gray. Each data point identified in this way was reviewed and professional judgment was used to decide if the value should be retained or excluded. In order to avoid inappropriate biases, blood lead outlier designations were restricted to values that were clearly aberrant from a time-course and/or dose-response perspective. Values which were excluded are identified by a heavy black box outlining the values. Rationale for outlier exclusion is provided in Table A-5.

2.3 Blood Lead AUC

The area under the blood lead vs time curve for each animal was calculated by finding the area under the curve for each time step using the trapezoidal rule:

$$AUC(d_i \text{ to } d_j) = 0.5*(r_i+r_j)*(d_j-d_i)$$

where:

d = day number
r = response (blood lead value) on day i (r_i) or day j (r_j)

The areas were then summed for each of the time intervals to yield the final AUC for each animal. These calculations are shown in Table A-6. If a blood lead value was missing (either because of problems with sample preparation, or because the measured value was excluded as an outlier), the blood lead value for that day was estimated by linear interpolation.

2.4 Liver, Kidney, and Bone Lead Data

At sacrifice (day 15), samples of liver, kidney, and bone (femur) were removed and analyzed for lead. The raw data (expressed as ug Pb/L of prepared sample) are summarized in Table A-7. These data were adjusted as follows: a) non-detects were evaluated by assuming a value equal to one-half the quantitation limit, and b) the concentrations in prepared sample were converted to units of concentration in the original biological sample by dividing by the following factors:

Liver:	0.1 kg wet weight/L prepared sample
Kidney:	0.1 kg wet weight/L prepared sample
Bone:	2 gm ashed weight/L prepared sample

The resulting values are shown in the right-hand column of Table A-7.

3.0 CURVE FITTING

Basic Equations

A commercial curve-fitting program (Table Curve-2D™ Version 2.0 for Windows, available from Jandel Scientific) was used to derive best fit equations for each of the individual dose-response data sets derived above. A least squares regression method was used for both linear and non-linear equations. As discussed in the text, three different user-defined equations were fit to each data set:

Linear (LIN): $\text{Response} = a + b \cdot \text{Dose}$

Exponential (EXP): $\text{Response} = a + c \cdot (1 - \exp(-d \cdot \text{Dose}))$

Combination (LIN+EXP): $\text{Response} = a + b \cdot \text{Dose} + c \cdot (1 - \exp(-d \cdot \text{Dose}))$

Constraints

In the process of finding the best-fits of these equations to the data, the values of the parameters (a, b, c, and d) were constrained as follows:

- Parameter "a" (the intercept, equal to the baseline or control value of the measurement endpoint) was constrained to be non-negative and was forced in all cases to be the same for the reference material (lead acetate) and the test materials. This is because, by definition, all dose-response curves for groups of animals exposed to different materials must arise from the same value at zero dose. In addition, for blood lead data, "a" was constrained to be equal to the mean of the control group ± 20% (typically 7.5 ± 1.5 AUC units).

- Parameter "b" (the slope of the linear dose-response line) was constrained to non-negative values, since all of the measurement endpoints evaluated are observed to increase, not decrease, as a function of lead exposure.

- Parameter "c" (the plateau value of the exponential curve) was constrained to be non-negative, and was forced to be the same for the reference material (lead acetate) and the test material. This is because: 1) it is expected on theoretical grounds that the plateau (saturation level) should be the same regardless of the source of lead, and 2) curve-fitting of individual curves tended to yield values of "c" that were close to each other and were not statistically different.

- Parameter "d" (which determines where the "bend" in the exponential equation occurs) was constrained to be greater than 0.0045 for the lead acetate blood lead (AUC) dose-response curve. This constraint was judged to be necessary because the weight of evidence from all studies clearly showed the lead acetate blood lead dose response curve was non-linear and was best fit by an exponential equation, but in some studies there were only two low doses of lead acetate used to define the dose-response curve, and this narrow range data set could sometimes be fit nearly as well by a linear as an exponential curve. The

choice of the constraint on "d" was selected to be slightly lower than the observed best-fit value of "d" (0.006) when data from all lead acetate AUC dose-response curves from all of the different studies in this program were used. This approach may tend to underestimate relative bioavailability slightly in some studies (especially at low dose), but use of the information gained from all studies is judged to be more robust than basing fits solely on the data from one study.

In general, one of these models (the linear, the exponential, or the combination) usually yielded a fit (as judged by the value of the adjusted correlation coefficient R^2 and by visual inspection of the fit of the line through the measured data points) that was clearly superior to the others. If two or more models fit the data approximately equally well, then the simplest model (that with the fewest parameters) was selected.

Outlier Identification

During the dose-response curve fitting process, all data were carefully reviewed to identify any anomalous values. Typically, the process used to identify outliers was as follows:

Step 1 — Any data points judged to be outliers based on information derived from analysis of data across multiple studies (as opposed to conclusions drawn from within the study) were excluded (*a priori* outliers).

Step 2 — The remaining raw data points were fit to the equation judged to be the most likely to be the best fit (linear, exponential, or mixed). Table Curve 2-D was then used to plot the 95% prediction limits around the best fit line. All data points that fell outside the 95% prediction limits were considered to be outliers and were excluded.

Step 3 — After excluding these points (if any), a new best-fit was obtained. In some cases, data points originally inside the 95% prediction limits were now outside the limits. However, further iterative cycles of data point exclusion were not performed, and the fit was considered final.

It should be noted that professional judgment can be imposed during any stage of the above outlier identification process. Table A-8 shows outliers selected using professional judgement.

Curve Fit Results

Table A-8 lists the data used to fit these curves, indicating which endpoints were excluded as outliers and why. Table A-9 shows the type of equation selected to fit each data set, and the best fit parameters. The resulting best-fit equations for the data sets are shown in Figures A-4 to A-15. Values excluded as outliers are represented in the figures by the symbol "+".

4.0 RESULTS -- CALCULATED RBA VALUES

The value of RBA for a test substance was calculated for a series of doses using the following procedure:

1. For each dose, calculate the expected response to test material, using the best fit equation through the dose-response data for that material.

2. For each expected response to test material, calculate the dose of lead acetate that is expected to yield an equivalent response. This is done by "inverting" the dose-response curve for lead acetate, solving for the dose that corresponds to a specified response.

3. Calculate RBA at that dose as the ratio of the dose of lead acetate to the dose of test material. For the situation where both curves are linear, the value of RBA is the ratio of the slopes (the "b" parameters). In the case where both curves are exponential and where both curves have the same values for parameters "a" and "c", the value of RBA is equal to the ratio of the "d" parameters.

The results are summarized in Table A-10.

5.0 QUALITY ASSURANCE DATA

A number of steps were taken throughout this study and the other studies in this project to ensure the quality of the results, including 5% duplicates, 5% standards, a program of interlaboratory comparison. These steps are detailed below.

Duplicates

Duplicate samples were prepared and analyzed for about 5% of all samples generated during the study. Table A-11 lists the first and second values for blood, liver, kidney, and bone. The results are shown in Figure 3-1 in the main text.

Standards

The Centers for Disease Control and Prevention (CDCP) provides a variety of blood lead "check samples" for use in quality assurance programs for blood lead studies. Each time a group of blood samples was prepared and sent to the laboratory for analysis, several CDCP check samples of different concentrations were included. Table A-12 lists the concentrations reported by the laboratory compared to the nominal concentrations indicated by CDCP for the samples submitted during this study, and the results are plotted in Figure 3-2 in the main text.

APPENDIX B

WASHINGTON GROUP MEMORANDUM